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Ny dansk patentansøgning
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Tissue inhibitor of matrix metalloproteinases type-1 (TIMP-1) as a marker of cancer
Vor ref: 20236 DK 1

Plougmann, Vingtoft & Partners

TISSUE INHIBITOR OF MATRIX METALLOPROTEINASES TYPE-1 (TIMP-1) AS A MARKER OF CANCER

BRIEF DESCRIPTION OF THE INVENTION

The present invention relates to a biochemical test to be used to screen large populations for the occurrence of cancer. In particular, the invention relates to the screening for the occurrence of early colorectal cancer, but additional data suggest that the invention will also be useful for screening for other cancer types, e.g. breast cancer, while non-malignant diseases such as inflammatory bowl diseases do not give false positive results, suggesting a high specificity of the test.

The biochemical test is based on the measurement of tissue inhibitor of metalloproteinases type 1 (TIMP-1), in various body fluids, including plasma, serum and urine. TIMP-1 concentration can be determined either as the total TIMP-1 concentration, the concentration of complexes between TIMP-1 and MMP-9, the concentration of free TIMP-1 and/or ratios and fractions thereof. According to the invention, individuals with a high likelihood of having cancer, e.g. colorectal cancer, can be identified by their elevated levels of TIMP-1 in their blood or urine, while individuals with low levels of these factors are unlikely to suffer from cancer, e.g. colorectal cancer. Thus, the invention can be used to identify individuals with a high probability of having early, non-symptomatic cancer. The identified individuals should be further examined and if cancer is found, the patients should subsequently be offered surgery and/or irradiation, and/or adjuvant anti-neoplastic therapy, thereby increasing the chance of cure of cancer for the individual.

BACKGROUND

Matrix metalloproteases (MMP's) play a pivotal role in cancer growth and spread, contributing to enzymatic degradation of the integrity of the extracellular matrix (Liotta *et al*, 1991; Stetler-Stevenson *et al*, 1993; MacDougall & Matrisian, 1995). The naturally occurring inhibitors of MMP's - tissue inhibitors of MMP's (TIMP's) - form tight 1:1 stoichiometric complexes with the activated forms of the MMP's (Welgus *et al*, 1985; Kleiner *et al*, 1993) thereby inhibiting the catalytic activity of these enzymes (Stetler-Stevenson *et al*, 1996; Goldberg *et al*, 1992; Birkedal-Hansen *et al*, 1993). In support

of a protective rol of TIMP-1 and TIMP-2, it was found that *in vitro* invasiveness (Khokha *et al*, 1992a; Khokha & Waterhouse, 1993) and metastatic spread of tumour cells in experimental animal models (Khokha *et al*, 1992b; DeClerck *et al*, 1992) were inhibited by transfection of cancer cells with the genes encoding either TIMP-1 or TIMP-2. While the balance between the matrix degrading properties of MMP's and the inhibitory effect of TIMP's is closely regulated under normal physiological conditions (Matrisian, 1992; Thorgeirsson *et al*, 1993; Birkedal-Hansen *et al*, 1993), this balance might be disrupted in malignant tissue.

A number of enzyme linked immunoassays for detection of TIMP-1 (Kodama *et al*, 1989; Cooksley *et al*, 1990; Clark *et al*, 1991) and TIMP-2 (Fujimoto *et al*, 1993) have been reported. These assays have been applied to various forms of body fluids, e.g. serum, plasma, amniotic fluid, cerebrospinal fluid, urine, but the number of samples tested has not been sufficient to establish normal ranges for the plasma level of the TIMP's in healthy individuals (Kodama *et al*, 1989; Clark *et al*, 1991). Furthermore, recovery of specific signal from clinical samples has not been demonstrated with internal standards.

In a study by Mimori et al (Mimori *et al*, 1997) in which tumour tissue levels of TIMP-1 mRNA were studied in patients with gastric carcinoma, high tumour/normal tissue ratios of TIMP-1 mRNA were found to be associated with increased invasion and poor prognosis. ELISA studies of TIMP-1 in serum from prostate cancer patients (Baker *et al*, 1994) showed a high degree of overlap when compared to serum TIMP-1 levels in control individuals. In another study of prostate cancer, plasma TIMP-1 levels were not significantly different between patients with localized prostate cancer and control subjects (Jung *et al*, 1997).

Studies of TIMP-1 in complex with MMP-9 in plasma of patients with advanced gastrointestinal cancer and gynecological cancer (Zucker *et al*, 1995) have demonstrated that the complex was found in significantly higher levels in blood samples from cancer patients as compared to healthy control individuals, and that patients with high levels of TIMP-1:MMP-9 complex had a shorter survival (Zucker *et al*, 1995). However, this study did not include measurements of total TIMP-1. Furthermore, in this study no differences in complex levels were found between patients with breast cancer and control individuals.

DETAILED DESCRIPTION OF THE INVENTION

In a number of cancer types, there is a critical and unmet need for highly sensitive and specific markers for screening of large populations for the presence of malignant disease. Such markers should be used to screen individuals for the occurrence of early cancer, and those with a high likelihood of cancer should be further examined and if cancer is found, the patients should subsequently be offered surgery, and/or irradiation, and/or adjuvant anti-neoplastic therapy.

Since proteinases and their receptors and inhibitors seem to play a pivotal role in the basic mechanisms leading to cancer invasion, these molecules may be expressed at a very early time point in the carcinogenic process. Since many of these molecules exert their biological action extracellularly, they may be present at elevated levels in body fluids even in patients with early stage invasive malignant diseases. Moreover, since these molecules are involved in the more basic features of the malignant progression, e.g. invasion and metastasis, it is most likely that patients with different types of cancers will share a similar increase in these molecules.

The present invention relates to a method to aid in the diagnosis of cancer in a patient, said method comprising determining the amount of total, complexed forms and/or free tissue inhibitor of metalloproteinases type 1 (TIMP-1) in body fluids such as a blood sample.

The method can be applied to an unselected population, but more efficiently to a population already identified as having an increased risk of developing cancer, e.g. individuals with genetic disposition, individuals who have been exposed to carcinogenic substances, and individuals with cancer predisposing non-malignant diseases. In the case of colorectal cancer, the population selected for the TIMP-1 assay could represent individuals with a prior polyp, individuals with Crohn's disease or Colitis Ulcerosa, individuals with one or more family members with colorectal cancer and individuals with prior resection of an early colorectal cancer.

When an individual has been identified as having high TIMP-1 levels in his or her body fluid, the individual should be referred for further examination. If a cancer is found, the

patient could be offered surgery, irradiation and/or adjuvant anti-neoplastic therapy aiming at curing the patient of the canc r.

Highly sensitive and specific TIMP-1 assays have to be developed and rigorously tested and validated before such an analysis can be performed on a routine basis. Example 1 describes the development and validation of an assay that measures total TIMP-1 with high sensitivity and specificity. It is described that healthy blood donors have a very narrow range of plasma total TIMP-1.

In Example 2 the formatting of a TIMP-1:MMP-9 ELISA is described. This format and execution of this assay follow the assay for total TIMP-1, except that a polyclonal antibody against MMP-9 is used in the detecting step. By substituting the MMP-9 antibody with an antibody against another MMP, complexes between TIMP-1 and this other MMP can be quantitated.

In addition, by subtracting the complexed TIMP-1 from the total TIMP-1, the concentration of free TIMP-1 can be determined. In this case, free TIMP-1 is defined as the fraction of the total TIMP-1 that is not in complex with the MMP in question. Furthermore, various fractions and ratios between total, complexed and/or free TIMP-1 can be calculated and tested for their association to patient diagnosis.

In Example 3 it is shown that patients suffering from colorectal cancer have significantly elevated total TIMP-1 levels in their preoperatively sampled plasma. By performing a percentile plot including the total TIMP-1 levels from the colorectal cancer patients as well as total TIMP-1 levels found in plasma samples obtained from healthy blood donors, it could be shown that at a TIMP-1 concentration of 91,8 microgram/L, which is the 90th centile of the healthy donors, 90% of the cancer patients could be identified as having increased plasma TIMP-1 levels. Thus, using this cut-off value, it could be shown that total TIMP-1 measurements in plasma could identify 90% of the cancer patients (diagnostic sensitivity) with a 90% diagnostic specificity (10% of the healthy blood donors were classified as being high). If a higher or lower sensitivity/specificity is desired, the cut-off value can be changed upwards or downwards. This is illustrated in Figure 9 showing a ROC curve of TIMP-1 in colorectal cancer patients versus healthy blood donors. All figures which can be deducted from this ROC curve are within the scope of th pres nt invention.

The clinical value of a biochemical marker for cancer screening is related to its ability to detect early stages of the cancer disease, making it likely that early detection will have an impact on the survival of the individual. It could be shown that total TIMP-1 was as efficient a screening marker in early colorectal cancer (Duke's stage B disease) as it was in the total population of colorectal cancer patients (Figure 10), indicating that screening with total TIMP-1 could result in more patients being diagnosed with early stage cancer, which by itself will result in a better survival. In a similar manner, all figures which can be deducted from this ROC curve are within the scope of the present invention.

The specificity of a given cancer screening test is based on the efficiency of the test to identify only those patients suffering from cancer while patients suffering from non-malignant diseases should not be identified as false positive subjects. In the case of colorectal cancer, it is important that the test in question can distinguish between malignant and non-malignant diseases of the colon and rectum. This is particularly important for diseases like Crohn's disease and Colitis Ulcerosa since patients with these diseases are in a higher risk of developing cancer and should therefore be candidates for the screening procedure. In Example 4 it is shown that total TIMP-1 levels are significantly higher in patients with colorectal cancer than in patients with inflammatory bowl diseases, suggesting that total TIMP-1 can be used to screen for colorectal cancer in a population of patients with inflammatory bowl diseases. That TIMP-1 is not increased in non-malignant diseases is supported by a recent paper by Keyser (Keyser *et al*, 1999), demonstrating that patients with rheumatoid arthritis do not have increased plasma TIMP-1 levels. In Example 6 total TIMP-1 values are described in patients with breast cancer as compared with total TIMP-1 levels in healthy female blood donors. It was shown that the patients had significantly higher plasma TIMP-1 levels than the blood donors. Since proteases, their receptors and inhibitors are causally involved in the basic processes leading to cancer invasion and metastasis, it is contemplated that the present finding of elevated TIMP-1 levels in patients with colorectal cancer and in patients with breast cancer can be extended to patients with other forms of solid tumors, such as lung cancer, gastric cancer, ovarian cancer, prostate cancer, cervical cancer and liver cancer.

TIMP-1 is known to exist either as the free molecule or in complex with MMP's, preferentially MMP-9. In analogy with prostate specific antigen, it is contemplated that measuring total TIMP-1, complexed TIMP-1 and free TIMP-1 will make it possible to validate each of these fractions for their potential diagnostic value. In addition, it will be possible to calculate ratios between the different fractions which will have diagnostic value.

Since TIMP-1 was found to be elevated in patients with colorectal cancer and in patients with advanced breast cancer (see below), and since proteases and their inhibitors seem to play a role in the basic mechanisms of cancer invasion, it is contemplated that patients with other types of solid tumours, e.g. prostate, lung, gastric, ovarian, cervical and liver cancer, will also have elevated plasma levels of TIMP-1. Thus, the diagnostic value of total TIMP-1, complexed TIMP-1 and free TIMP-1 as well as ratios between the various fractions in other types of cancer should be evaluated in a similar manner.

FIGURE LEGENDS

Figure 1: Kinetic ELISA for TIMP-1. Progress curves for the change in absorbance at 405 nm produced by hydrolysis of p-nitrophenyl phosphate by solid-phase bound alkaline phosphatase immunoconjugate. The data shown are generated by 4 individual assay wells treated with 4 different concentrations of purified recombinant TIMP-1; 10 μg/L (∇-∇), 2.5 μg/L (Δ-Δ), 0.63 μg/L (□-□) and 0.16 μg/L (O-O). The lines shown have been fitted by simple linear regression.

Figure 2: TIMP-1 standard curve. ELISA well absorbance measurements for triplicate TIMP-1 standards in the range of 0.0 to 5 μg/L are collected automatically over 60 minutes, with readings taken at 405 nm every 10 min. Progress curves are computed for each assay well and the rates thus obtained are fitted to a standard curve using a four-parameter equation of the form $y = d + [(a-d)/(1+(x/c)^b)]$. In the example shown, the four derived parameters had the following values: a=1.87, b=1.11, c=3.35, d=73.5. The correlation coefficient for the fitted curve is >0.999.

Figure 3: Recovery of ELISA signal from standard TIMP-1 added in increasing concentration to assay dilution buffer (□-□), a 1:100 dilution of EDTA plasma pool (Δ-Δ), a 1:100 dilution of citrate plasma pool (∇-∇) and a 1:100 dilution of heparin plasma pool

(O-O). The values shown are the means of triplicates. The correlation coefficient for each fitted curve is greater than 0.99.

Figure 4: Western blotting of immunoabsorbed patient plasma sample. Lane 1: standard TIMP-1; lane 2: eluate of patient citrate plasma sample diluted 1:10 and immunoabsorbed with sheep polyclonal anti-TIMP-1. Bands of unreduced standard TIMP-1 and TIMP-1 isolated from plasma sample both appear just below 30 kDa.

Figure 5a: Percentiles plot for the level of TIMP-1 (µg/L) measured by ELISA in citrate plasma (O) and EDTA plasma (Δ) from the same individual in a set of 100 volunteer blood donors.

Figure 5b: Linear regression plot for the level of TIMP-1 in citrate plasma samples compared with EDTA plasma samples from the same 100 individuals. The equation of the fitted line is y = 0.93x, with a regression coefficient of 0.99.

Figure 6: Percentiles plot for the level of TIMP-1 (µg/L) measured by ELISA in two sets of citrate plasma samples obtained by the same procedure from volunteer blood donors at different times. 100 samples from May =97 (Δ) and 94 samples from Sept =96 (□).

Figure 7: Percentile plot for the level of TIMP-1 (µg/L) measured by ELISA in 194 citrate plasma samples from volunteer blood donors and divided by sex into 107 males (Δ) and 87 females (O).

Figure 8: Percentiles plot for the level of TIMP-1 (µg/L) measured by ELISA in 591 EDTA plasma samples from colorectal cancer patients (O) and 100 healthy blood donors (Δ).

Figure 9: ROC curve for 591 colorectal cancer patients and 100 healthy blood donors. X-axis: 1-specificity.

Figure 10: ROC curve for 219 colorectal cancer patients with Duke's stage B disease and 100 healthy blood donors. X-axis: 1-specificity.

Figure 11: Percentiles plot for the level of total TIMP-1 (µg/L) measured by ELISA in 19 EDTA plasma samples from female breast cancer patients (O) and 87 healthy blood donors (Δ).

Figure 12: Box plot of the level of TIMP-1 (µg/L) measured by ELISA in healthy donors, patients with Crohn's disease, patients with Ulcerative Colitis and colorectal cancer patients.

EXAMPLES

Example 1

Development of an ELISA to quantitate total TIMP-1 concentrations in human plasma.

This example describes the development and validation of an ELISA that measures total amounts of TIMP-1 in plasma. In addition, this Example provides information on the levels of total TIMP-1 in different plasma preparations as well as on the plasma levels of TIMP-1 in healthy blood donors of both sexes.

Materials and methods:

Blood donors

Blood samples were initially obtained from 94 volunteer blood donors, comprising 51 males aged 19 to 59 years (median: 41 years) and 43 females aged 20 to 64 years (median: 36 years). In another later collection 100 donor samples were obtained, comprising 56 males aged 19 to 59 years (median 42: years) and 44 females aged 20 to 60 years (median: 36.5 years). Donors gave blood on a volunteer basis and were all apparently healthy. Informed consent was obtained from all blood donors, and permission was obtained from the local Ethical Committees.

Blood collections and plasma separation

Peripheral blood was drawn with minimal stasis (if necessary a maximum of 2 min stasis with a tourniquet at maximum +2 kPa was accepted) into pre-chilled citrate, EDTA, or heparin collection tubes (Becton-Dickinson, Mountain View, CA), mixed by 5 times inversion, and immediately chilled on ice. As soon as possible (no later than 1.5 h after collection) the plasma was separated from blood cells by centrifugation at 4°C at 1,200 x g for 30 min, and stored frozen at -80°C prior to assay. Plasma pools were made with freshly collected samples from at least ten donors, aliquoted and stored frozen at -80°C. When analysed the samples were thawed quickly in a water bath at 37°C and placed on ice until the 1:100 plasma dilutions were made.

TIMP-1 ELISA

A sensitive and specific sandwich ELISA was developed, using TIMP-1 antibodies developed at the Strangeways Laboratories (Hembry *et al*, 1985). A sheep polyclonal anti-TIMP-1 antibody (Hembry *et al*, 1985; Murphy *et al*, 1991) was used for catching, a murine monoclonal anti-TIMP-1 IgG1 (MAC-15) (Cooksley *et al*, 1990) for detection of the antigen, and a rabbit anti-mouse immunoglobulins/alkaline phosphatase conjugate (Catalog number D0314, Dako, Glostrup, Denmark) enabled kinetic rate assay. The latter conjugate was supplied preabsorbed against human IgG, thus avoiding cross-reaction with IgG in the plasma samples. As the monoclonal detection antibody MAC-15 recognises both free TIMP-1 and TIMP-1 in complex with MMP's (Cooksley *et al*, 1990), the total TIMP-1 content of the measured sample captured by the sheep polyclonal anti-TIMP-1 antibody (Hembry *et al*, 1985) was determined by the ELISA.

96-well immunoassay plates (Maxisorp, Nunc, Roskilde, Denmark) were coated for 1 h at 37°C with 100 µL/well of polyclonal sheep anti-TIMP-1 (4 mg/L) in 0.1 mol/L carbonate buffer, pH 9.5. Then the assay wells were rinsed twice with 200 µL/well of Super-BlockJ solution (Pierce Chemicals, Rockford, IL) diluted 1:1 with phosphate-buffered saline (PBS). The immunoassay plates were stored for up to 14 days at -20°C. On the day of use the plates were thawed at room temperature and washed 5 times in PBS containing 1 g/L Tween 20. Wells were then treated for 1 h at 30°C with 100 µL/well of triplicate 1:100 dilutions of plasma made in a sample buffer consisting of 50 mol/L phosphate, pH 7.2, 0.1 mol/L NaCl, 10 g/L bovine serum albumin (Fraction V, Boehringer-Mannheim, Penzberg, Germany) and 1 g/L Tween 20. On every assay plate the first three columns of wells (each column consisting of 8 wells) were incubated with a series of standards, consisting of seven serial dilutions in triplicate of purified recombinant human TIMP-1, starting at 10 µg/L then 5, 2.5, 1.25, 0.625, 0.313 and 0.156 µg/L. Also included on each plate were triplicate blank wells containing only sample dilution buffer, and 2 sets of triplicate wells of a 1:100 dilution of a control citrate plasma pool; the first set of triplicate plasma pool was added as the first sample to the assay plate and the second set of triplicate plasma pool was added as the last. After TIMP-1 binding, the wells were washed 5 times, then treated for 1 h at 30°C with 100 µL/well of the purified murine monoclonal anti-TIMP MAC-15 (0.5 mg/L) in sample dilution buffer. After another 5 washes the wells were incubated for 1 h at 30°C with 100 µL/well of rabbit anti-mouse immunoglobulins/alkaline phosphatase conjugate diluted 1:2000 in

sample dilution buffer. Following 5 washes with washing solution and 3 washes with pure water, 100 µL of freshly made p-nitrophenyl phosphat (Sigma, St. Louis, MO) substrate solution (1.7 g/L in 0.1 mol/L Tris.HCl, pH 9.5, 0.1 mol/L NaCl, 5 mmol/L $MgCl_2$) was added to each well, and the plate was placed in a Ceres 900J plate reader (Bio-Tek Instruments, Winooski, VT). The yellow colour development at 23°C was monitored automatically, with readings taken at 405 nm against an air blank every 10 min for 60 min. KinetiCalc II software was used to manage the data, calculate the rate of colour change for each well (linear regression analysis) and compute from the rates for the TIMP-1 standards a 4-parameter fitted standard curve, from which the TIMP-1 concentration of each plasma sample was calculated.

Recovery experiments

The recovery of signal from standard TIMP-1 was measured after addition to 1:100 dilutions of citrate, EDTA or heparin plasma pools. Standard TIMP-1 was added to these plasma pool solutions to give final concentrations in the range of 0 to 10 µg/L. The recovery in each case was calculated from the slope of the line representing TIMP-1 signal as a function of concentration, where 100% recovery was defined as the slope obtained when TIMP-1 was diluted in the sample dilution buffer.

Immunoblotting

Citrate plasma from a patient with a high level of TIMP-1 in blood (634 µg/L, determined by ELISA) was diluted 1:10 and added to a column of protein A-Sepharose containing polyclonal sheep anti-TIMP-1. Following 5 times recycling, bound proteins were eluted from the column and SDS-gel electrophoresis of 50 µL of the resulting eluate was carried out using a 12% acrylamide Ready GelJ (Bio-Rad). 15 µL of a mixture of low molecular weight (Pharmacia) and high molecular weight markers (Bio-Rad) and 50 µL of TIMP-1 standard (100 µg/L) in Laemmli Sample BufferJ were also run on the gel. Proteins were transferred electrophoretically from the gel onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was incubated for 1 h at room temperature with 1% skimmed milk powder in TBS. After washing, the membrane was incubated for 1 h at room temperature with 20 ml of MAC-15 at a concentration of 5 mg/L followed by washing and incubation for another hour at room temperatur with 20 ml of rabbit anti-mouse immunoglobulins/alkaline phosphatase conju-

gat diluted 1:1000. Finally the membrane was washed, and phosphate substrate solution (NBT/BCIP) was added to develop colour.

Results:

ELISA performance

Development of colour in each well was a linear function of time for all concentrations of TIMP-1 measured in these experiments (Figure 1), with correlation coefficients for the automatically fitted lines typically better than 0.99. The standard curve for the rates plotted against the TIMP-1 concentration consisted of the linear and upper curved regions (over the range of 0 to 5 μg/L) of a sigmoidal curve, and the correlation coefficient for the 4-parameter fit was typically better than 0.999 (Figure 2). The rate with no TIMP-1 (read against air) was 1.21±0.15 (mean±SD) milliabsorbance units/min (n=29), while the rate with 10 μg/L standard TIMP-1 was 50.3±6.01 milliabsorbance units/min (n=29). The limit of detection for the assay, defined as the concentration of TIMP-1 corresponding to a signal 3 SD above the mean for the TIMP-1 blank, was 0.089 μg/L or 13% of the mean of the measured concentration of TIMP-1 in healthy citrate plasma samples diluted 1:100. The intra-assay coefficient of variation for 16 replicates of a control citrate plasma pool measured on the same plate was 5.3%, and the inter-assay coefficient of variation for 29 successive assays of the plasma pool (on different days) was 6.2%. This plasma pool had a TIMP-1 content of 57.8 μg/L, corresponding to the 22nd centile of the plasmas subsequently measured.

Recovery of recombinant TIMP-1 after dilution in plasma

Specific signal recovery was determined by addition of increasing concentrations of purified TIMP-1 standard to a fixed 1:100 dilution of plasma pool and subsequent measurement of the ELISA signal. In diluted citrate plasma pool 104% recovery was obtained, 101% in diluted EDTA plasma pool and 87% in diluted heparin plasma pool (Figure 3). Thus the recovery of TIMP-1 signal from an internal standard was acceptable for all preparations of plasma, but recovery from EDTA and citrate plasma was more complete than heparin plasma.

Dilution curves for plasma TIMP-1 signal

Serial dilutions of citrate, EDTA and heparin plasma pools were made and TIMP-1 levels assayed to test for linear reduction in ELISA signal. Citrate, EDTA and heparin plasmas all gave good linearity of signal as a function of dilution. The 1% plasma dilution which was chosen for subsequent determinations lay well within the range of this linear dilution curve.

Immunoblotting of plasma TIMP-1

The Western blot of the immunoabsorbed patient plasma sample showed a clear band of 28 kDa (Figure 4, lane 2), corresponding to free, uncomplexed TIMP-1 (Figure 4, lane 1). No bands were found at the expected higher molecular weights corresponding to complexes between MMP's and TIMP-1, e.g. MMP-2:TIMP-1, 100 kDa. This could indicate either that the majority of TIMP-1 was present in the plasma as the free form, or that complexes were dissociated during SDS-PAGE. Although the sample was left both unreduced and unheated in order to preserve any complexes present in the plasma sample, it has been reported that MMP:TIMP complexes may be unstable in SDS-PAGE (Wilhelm *et al*, 1989; Stetler-Stevenson *et al*, 1989; Moll *et al*, 1990), even under non-reducing SDS-PAGE conditions (Moutsiakis *et al*, 1992).

TIMP-1 in citrate and EDTA plasma from the same healthy donor

A collection of citrate and EDTA plasma samples taken simultaneously from 100 healthy donors was available for this study. These samples were not specifically collected as platelet poor plasma. However, a small representative number of samples prepared as platelet poor plasma did not differ significantly in TIMP-1 plasma values as found below. The percentile plots for TIMP-1 levels determined in these samples are shown in Figure 5a. The values in each set approximated a normal distribution; the citrate plasma TIMP-1 levels had a reference range (10th to 90th percentile) of 55.0 to 90.3 μg/L and a mean of 69.2±13.1 μg/L. Similarly, the reference range for the EDTA plasma TIMP-1 levels was from 58.0 to 91.8 μg/L and the mean was 73.5±14.2 μg/L. For both citrate and EDTA plasma, the mean TIMP-1 levels were in close proximity to

the median levels (Table 1). A paired means comparison showed that the level of TIMP-1 in citrate plasma was significantly lower by 4.34 μg/L (95% CI 2.34-6.33; p<0.0001) than the level in EDTA plasma from the same individual. However, it is likely that this reflected the difference in sampling procedure when collecting EDTA and citrate plasma from the donors. EDTA plasma tubes contained dry anticoagulant material, while citrate plasma tubes contained a small amount of liquid citrate buffer which gave a small and variable systematic dilution error (x 9/10). The level of TIMP-1 in citrate plasma correlated with EDTA plasma from the same individuals: the linear regression plot in Figure 5b shows a regression coefficient of 0.99 and the slope of the fitted line is 0.93, illustrating the small dilution error. A non-parametric Spearman's rank test for the data set gave an rho value of 0.62 and p<0.0001.

TIMP-1 levels in citrate plasma

In total, 194 citrate plasma samples from healthy blood donors were assayed, comprising 94 samples taken from one collection and 100 samples taken 9 months later from a different set of donors. Figure 6 shows the percentile plots for TIMP-1 levels measured in these two independent groups. The reference range (10th to 90th percentile) for TIMP-1 levels in citrate plasma from the first collection was 53.3 to 77.7 μg/L with a mean TIMP-1 level of 65.4±10.1 μg/L which was indistinguishable from the median (Table 1); the values approximating a normal distribution. The mean TIMP-1 level for the second collection was 69.2±13.1 μg/L (reference range: 55.0 to 90.3 μg/L). An unpaired means comparison showed that TIMP-1 levels in the two sets of citrate plasma samples taken in the two different collections differed only by 3.82 μg/L (95% CI: 0.50-7.14 μg/L; p=0.024). Moreover, no significant difference was apparent between the plasma pool controls (n=8 in each set) included in each set of assays (mean difference 0.36 μg/L; 95% CI: 1.71-2.44 μg/L; p=0.69). The mean TIMP-1 level for the total material of 194 citrate plasma samples was 67.3±11.8 μg/L, which was close to the median of 66.1 μg/L, the levels approximating a normal distribution (reference range 54.0 to 82.7 μg/L).

TABLE 1:

SUMMARY OF TIMP-1 LEVELS DETERMINED IN BLOOD FROM HEALTHY DONORS:

Blood fraction	Date of sampling	Number of samples	Mean±SD (µg/L)	Median (µg/L)	Reference range* (µg/L)
Citrate plasma	Sept. 96	94	65.4±10.1	65.6	53.3-77.7
Citrate plasma	May 97	100**	69.2±13.1	67.0	55.0-90.3
Citrate plasma	96+97	194	67.3±11.8	66.1	54.0-82.7
EDTA plasma	May 97	100**	73.5±14.2	71.2	58.0-91.8

*The reference range is defined as between the 10th and 90th percentiles.

**These samples were collected from the same donors.

Tests for correlations to sex and age of the donor

In the assays performed to obtain these results, the control plasma values measured in the same assays had a coefficient of variation of 2.7%. Percentiles for TIMP-1 levels in 194 citrate plasma samples divided according to sex are shown in Figure 7. The mean TIMP-1 value for 107 male donors was 70.4±12.0 µg/L (median 69.4 µg/L) with a reference range (10th to 90th percentile) from 56.2 to 86.6 µg/L, while the mean TIMP-1 value for 87 female donors in this set was 63.5±10.5 µg/L (median: 62.0 µg/L) with the reference range from 51.8 to 77.0 µg/L. There was a significant difference ($p<0.0001$) in TIMP-1 mean levels between the two groups; males were higher by 6.91 µg/L than females (95% CI 3.67-10.14 µg/L, unpaired means comparison). There was a trend towards an increase in plasma TIMP-1 with increasing age (Spearman's rho=0.33, P=0.0011), but this was not stronger when the material was divided accord-

ing to sex (females: Spearman's rho=0.29, P=0.006; males: Spearman's rho=0.35, P=0.0003). In the EDTA plasma donor material, the mean TIMP-1 value for 56 males was 76.9±15.0 µg/L (median: 75.1 µg/L) with a reference range from 58.8 to 96.9 µg/L while 44 female donors had a mean TIMP-1 plasma level of 69.3±11.8 µg/L (median: 67.9) with a reference range from 56.1 to 85.5 µg/L. Again, a significant difference in TIMP-1 means appeared between males and females, with males higher by 7.53 µg/L than females (95% CI 2.04-13.0, p=0.0076, unpaired means comparison).

Discussion:

The assay described above enabled accurate determination of total TIMP-1 in human plasma samples. Detection of captured TIMP-1 with MAC-15 was conveniently followed by incubation with a rabbit anti-mouse immunoglobulins/alkaline phosphatase conjugate, which allowed kinetic rate assays of the bound antigen. This permitted automated fitting of rate curves which has proven considerably more reliable than single end-point measurements. The use of a rapid blocking agent and a dilution buffer with high buffering capacity also facilitated reproducible assays. Including these elements in the final assay, requirements of sensitivity, specificity, stability, and good recovery of an internal standard were fulfilled.

The quantitative studies of TIMP-1 in blood from healthy donors showed that both citrate and EDTA plasma samples are suitable for the ELISA determination. Compared to other published ELISA studies of TIMP-1 in healthy donors' plasma (Jung *et al*, 1996; Fung *et al*, 1996), the levels of TIMP-1 found in the present study fell within a very narrow range. Some studies have reported values for serum, but plasma was selected for the present study to avoid the variable contribution of platelet activation to the measured TIMP-1 values (Cooper *et al*, 1985). While the plasma samples used in this study were not specifically prepared as platelet poor plasma, it is not anticipated from tests carried out that this would significantly change the values. The donor material was large enough to show that TIMP-1 levels in healthy plasma (both EDTA and citrate) approximated a normal distribution, for females as well as for males. For both EDTA and citrate plasma, the mean TIMP-1 levels were approx. 10% higher in males than in females. One explanation for the higher levels of inhibitor in the blood of males

is a higher release rate of TIMP-1 into blood from activated platelets, reflecting a tendency towards higher incidence of thromboembolic disease in the male population. When the males and females were considered separately, there was a weak correlation between TIMP-1 level and age as seen for the whole population (see above).

Example 2

Development of an ELISA to quantitate TIMP-1:MMP-9 complexes in plasma.

The following example describes an example of an assay that determines the concentration of TIMP-1:MMP-9 complexes in body fluids. The assay is applied to plasma samples of healthy blood donors in order to establish normal ranges of this complex.

Materials and methods:

TIMP-1:MMP-9 complex ELISA

A sensitive and specific sandwich ELISA was developed, using a TIMP-1 antibody developed at the Strangeways Laboratories, England (Hembry *et al*, 1985) and a MMP-9 polyclonal antibody developed in the Hematological Department, Rigshospitalet, Denmark (Kjeldsen *et al*, 1992). A murine monoclonal anti-TIMP-1 IgG1 (MAC-15) (Cooksley *et al*, 1990) was used for catching, a rabbit polyclonal anti-MMP-9 antibody was used for detection of the antigen, and a mouse anti-rabbit-immunoglobulins/alkaline phosphatase conjugate (Dako, Glostrup, Denmark) enabled kinetic rate assay. The latter conjugate was supplied preabsorbed against human IgG, thus avoiding cross-reaction with IgG in the plasma samples. As the monoclonal catching antibody MAC-15 captured both free TIMP-1 and TIMP-1 in complex with MMP's (Cooksley *et al*, 1990), and MMP-9 was recognized by the rabbit polyclonal anti-MMP-9 antibody, only TIMP-1:MMP-9 complexes present in the assayed sample were determined by the ELISA.

To prevent TIMP-1:MMP complex formation to form *ex vivo* during the sampling and assay procedures, a protease inhibitor (ie. Galardin, Batimastat, Marimastat) was added to the plasma sample after thawing. The addition of the proteas inhibitor ex-

cluded *in vitro* complex formation by inhibition of the catalytic activity of the active sit of the metalloproteases.

96-well immunoassay plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 µL/well of the purified murine monoclonal anti-TIMP MAC-15 (1 mg/L) in 0.1 mol/L carbonate buffer, pH 9.5. Prior to use the assay wells were rinsed twice with 200 µL/well of SuperBlockJ solution (Pierce Chemicals, Rockford, IL) diluted 1:1 with phosphate-buffered saline (PBS) and washed 5 times in PBS containing 1 g/L Tween 20. Wells were then treated for 1 h at 30°C with 100 µL/well of triplicate 1:100 dilutions of plasma made in a sample buffer consisting of 50 mol/L phosphate, pH 7.2, 0.1 mol/L NaCl, 10 g/L bovine serum albumin (Fraction V, Boehringer-Mannheim, Penzberg, Germany) and 1 g/L Tween 20. On every assay plate the first three columns of wells (each column consisting of 8 wells) were incubated with a series of standards, consisting of seven serial dilutions in triplicate of purified TIMP-1:MMP-9 complex, starting at 10 µg/L then 5, 2.5, 1.25, 0.625, 0.313 and 0.156 µg/L. Also included on each plate were triplicate blank wells containing only sample dilution buffer, and 2 sets of triplicate wells of a 1:100 dilution of a control citrate plasma pool; the first set of triplicate plasma pool was added as the first sample to the assay plate, and the second set of triplicate plasma pool was added as the last. After TIMP-1:MMP-9 complex binding, the wells were washed 5 times, then treated for 1 h at 30°C with 100 µL/well of the polyclonal anti-MMP-9 antibody (0.5 mg/L) in sample dilution buffer. After another 5 washes, the wells were incubated for 1 h at 30°C with 100 µL/well of mouse anti-rabbit immunoglobulins/alkaline phosphatase conjugate diluted 1:2000 in sample dilution buffer. Following 5 washes with washing solution and 3 washes with pure water, 100 µL of freshly made p-nitrophenyl phosphate (Sigma, St. Louis, MO) substrate solution (1.7 g/L in 0.1 mol/L Tris.HCl, pH 9.5, 0.1 mol/L NaCl, 5 mmol/L $MgCl_2$) was added to each well and the plate was placed in a Ceres 900J plate reader (Bio-Tek Instruments, Winooski, VT). The yellow colour development at 23°C was monitored automatically, with readings taken at 405 nm against an air blank every 10 min for 60 min. KinetiCalc II software was used to manage the data, calculate the rate of colour change for each well (linear regression analysis) and compute from the rates for the TIMP-1:MMP-9 standards a 4-parameter fitted standard curve, from which the TIMP-1:MMP-9 complex concentration of each plasma sample was calculat d.

Recovery experiments

The recovery of signal from standard TIMP-1:MMP-9 complex was measured after addition to 1:100 dilutions of citrate, EDTA or heparin plasma pools. Standard TIMP-1:MMP-9 complex was added to these plasma pool solutions to give final concentrations in the range of 0 to 10 μg/L. The recovery in each case was calculated from the slope of the line representing TIMP-1:MMP-9 complex signal as a function of concentration, where 100% recovery was defined as the slope obtained when TIMP-1:MMP-9 complex was diluted in the sample dilution buffer.

Healthy donors

100 donor plasma samples were obtained, comprising 56 males aged 19 to 59 years (median 42: years) and 44 females aged 20 to 60 years (median: 36.5 years). Donors gave blood on a volunteer basis and were all apparently healthy. Informed consent was obtained from all blood donors, and permission was obtained from the local Ethical Committees.

Example 3

Diagnostic value of TIMP-1 in patients with colorectal cancer.

Total TIMP-1 was measured with the TIMP-1 ELISA described in Example 1 in plasma from 591 colorectal cancer patients and in plasma from 100 healthy individuals. The measured TIMP-1 values from healthy donors and cancer patients were compared.

Materials and methods:

Patients

591 patients undergoing elective surgery for histologically verified colorectal cancer were included in the study. Blood samples were obtained preoperatively with informed cons nt from all patients in accordance with the Helsinki d claration, and permission

was granted by the local ethical commitees of Hvidovre and Aalborg Hospitals. All patients had histologically verified adenocarcinoma of the colon or rectum. It was found that 59 (10%) patients could be classified as having Duke's stage A disease, 219 (37%) patients Duke's stage B, 170 (29%) patients Duke's stage C and 143 (24%) patients Duke's stage D. 338 tumors were colon cancers and 253 tumors were rectum cancers. Clinical data such as age, sex and survival after surgery were collected. The median age was 69 years (range 33-90 years) and there were 237 females and 354 males represented in the patient material.

Healthy donors

The same donor population as described in Example 2.

Blood samples

Blood samples (5 ml) were collected preoperatively from all patients on the day of their operation. Peripheral blood was drawn with minimal stasis and collected in EDTA anticoagulant tubes (Becton-Dickinson, Mountain View, CA) in accordance with a previously described protocol (Example 1) to ensure good TIMP-1 measurements.

TIMP-1 ELISA

TIMP-1 levels were measured in all EDTA plasma samples by the sandwich ELISA described in Example 1.

Results:

TIMP-1 levels in plasma

Using kinetic rate ELISA, TIMP-1 levels were determined in all patient and healthy donor plasma samples. All the plasma samples had measurable levels of TIMP-1, and the median TIMP-1 value for the colorectal cancer patients was 141.1 ng/ml (range 53.7 - 788.7 ng/ml). There was a statistically significant difference in TIMP-1 levels when the patient mat rial was split into Duke's stage, Duke's A being the lowest and

Duke's D th highest (Kruskal-Wallis test, P<0.0001). However, higher TIMP-1 levels were not restricted to advanced disease. No significant correlation was found between TIMP-1 and sex (p=0.97). The median TIMP-1 level in plasma from healthy donors was 73.5±14.2 µg/L with a range of 58.0 to 91.8 µg/L. There was a relatively weak but not significant correlation between age and TIMP-1 level among the blood donors.

Diagnostic value of TIMP-1

Figure 8 shows a percentile plot of the TIMP-1 levels of the cancer patients and of the blood donors. It is seen from this figure that 88% of the colorectal cancer patients had TIMP-1 levels above the 90th centile of the healthy donors. Thus, using a cut-off value of 91.8 microgram/L (90th centile of the blood donors), the diagnostic sensitivity will be 90% and the diagnostic specificity will be 90%. Using the measured total TIMP-1 levels in plasma from healthy donors and colorectal cancer patients, Receiver Operating Characteristics curves (ROC curves) were constructed to evaluate the diagnostic value of TIMP-1 in blood. As seen from Figure 9, the curve was steep indicating a high sensitivity and specificity of TIMP-1 as a marker for colorectal cancer. Furthermore, it appears from this figure that increasing the diagnostic sensitivity will result in a decrease in specificity and vice versa. Figure 10 shows the same ROC curve now including only patients with early colorectal cancer, i.e. Duke's stage B disease.

Discussion:

These data suggest that TIMP-1 measurements in plasma can be used as a screening procedure to identify patients with a high risk of having colorectal cancer. In particular, TIMP-1 was as efficient in identifying patients with early cancer (Duke's stage B) as identifying patients with advanced cancer.

Example 4

Quantitation of TIMP-1 in plasma from patients with inflammatory bowel diseases.

Patients

50 patients with IBD (Inflammatory Bowel Disease) were included in the study. 24 patients had Colitis Ulcerosa and 26 patients had Crohn's Disease. The TIMP-1 levels found in EDTA plasma from healthy blood donors and colorectal cancer patients (Examples 1 and 3) were included for comparison.

TIMP-1 ELISA

TIMP-1 levels were measured in all EDTA plasma samples by the sandwich ELISA described in Example 1.

Results:

The measured TIMP-1 values were as follows:

Table 2

	Colitis Ulcer (n=24)	Crohn (n=26)	IBD total (n=50)
Median TIMP-1 (ng/ml)	83.3	96.8	89.0
Range TIMP-1 (ng/ml)	54.9-226	39.7-334	39.7-334

	Healthy donors (n=100)	Colorect Ca (n=677)
Median TIMP-1 (ng/ml)	71.2	140
Range TIMP-1 (ng/ml)	49.0-121	53.7-789

There was no significant difference when TIMP-1 values from patients with Colitis Ulcerosa and Crohn's Disease wer compared (Mann-Whitney; P=0.332).

However, there was a significant difference between plasma TIMP-1 levels found in healthy donors and TIMP-1 levels in plasma from IBD patients (Mann-Whitney; $P<0.0001$).

A significant difference was also found when TIMP-1 levels in plasma from IBD patients were compared to TIMP-1 levels in plasma from colorectal cancer patients (Mann-Whitney; $P<0.0001$). For a graphical illustration, see Figure 12.

Discussion:

These results show that patients with colorectal cancer have significantly higher TIMP-1 plasma levels than patients with IBD, suggesting that TIMP-1 can be used as a highly sensitive and specific marker to distinguish between non-malignant and malignant disease of the bowel.

Example 5

Calculation of the ratio between complexed (TIMP-1:MMP-9) and total TIMP-1, and the concentration of free TIMP-1.

Employing the above mentioned ELISA's (Examples 1 and 2), plasma concentrations of total TIMP-1 and TIMP-1:MMP-9 complex are measured in healthy donors and cancer patients. From the measured values of TIMP-1:MMP-9 complex and total TIMP-1, the ratio between the two as well as the free fraction of TIMP-1 is calculated for each assayed plasma sample. The calculated ratios and fractions found in plasma of healthy donors are compared to the ratios found in plasma of cancer patients, and the diagnostic value of such determinations is calculated.

More specifically, total concentration of TIMP-1 is determined by the assay described in Example 1. Total concentration of TIMP-1:MMP-9 complexes is determined by the assay described in Example 2. In order to calculate the concentration of free TIMP-1, the determined concentration of TIMP-1:MMP-9 complexes is subtracted from the measured total concentration of TIMP-1.

Example 6

Diagnostic value of TIMP-1 in patients with breast cancer.

This example describes total TIMP-1 determinations in plasma obtained from patients with breast cancer.

Materials and methods:

Patients and blood donors

Blood was collected from 19 stage IV breast cancer patients (aged 45 to 70 years) at the Oncology Department, Herlev University Hospital, Copenhagen, and from 87 healthy female blood donors (Example 1).

TIMP-1 ELISA

TIMP-1 levels were measured in all EDTA plasma samples by the sandwich ELISAs described in Example 1.

Results:

TIMP-1 levels in plasma from patients with advanced breast cancer

TIMP-1 was measured in EDTA plasma samples from 19 breast cancer patients with stage IV disease. TIMP-1 levels in patients were compared with TIMP-1 levels in healthy female donor citrate plasma. The mean TIMP-1 level measured in the citrate plasma samples of the 19 breast cancer patients was 292±331 µg/L (median: 236 µg/L) compared to a mean TIMP-1 level of 63.5±10.5 µg/L (median: 62.0 µg/L) in citrate plasma samples from 87 healthy female donors with a reference range of 51.8 to 77.0 µg/L. A Wald-Wolfowitz runs test indicated a highly significant difference with $P<0.0001$ between patient TIMP-1 levels and those of healthy donors. Figure 11

shows a percentile plot of TIMP-1 levels in EDTA plasma from the 19 breast cancer patients and the TIMP-1 levels found in plasma from the 87 healthy female donors.

Discussion:

These data suggest that plasma TIMP-1 measurements can be used to screen populations for breast cancer.

Example 7

Calculations of the ratios between complexed (TIMP-1:MMP-9) and total TIMP-1, and the concentration of free TIMP-1 in healthy individuals, patients with non-malignant diseases and patients with cancer.

This example describes the measurement of total TIMP-1 and TIMP-1:MMP-9 complexes as well as the calculation of free TIMP-1 in plasma samples from blood donors and from patients with non-malignant or malignant diseases. In addition, this example describes the calculation of ratios between total TIMP-1, TIMP-1:MMP-9 and free TIMP-1 as a means to increase the diagnostic sensitivity and specificity of TIMP-1 measurements in plasma samples.

Materials and methods:

TIMP-1:MMP-9 complex values are measured using the TIMP-1:MMP-9 complex ELISA (Example 2), and total TIMP-1 concentrations are measured by the ELISA described in Example 1. The samples included are the same as those described in Example 1 and Example 3.

Using the calculations described in Example 5, the various ratios are calculated and the diagnostic sensitivity and specificity for each of these is determined.

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CLAIMS

1. A method for screening a population for cancer, comprising determining the concentration of TIMP-1 in body fluid samples from individual members of the population, and discriminating the members of the population whose body fluid samples contain high concentrations of TIMP-1 as members having a high likelihood of having cancer from the members of the population having low concentrations of TIMP-1 and thereby unlikely to have cancer.

2. A method according to claim 1, wherein the TIMP-1 determined is total TIMP-1.

3. A method according to claim 1, wherein the TIMP-1 determined is TIMP-1:MMP complexes.

4. A method according to claim 3, wherein the TIMP-1:MMP complex is TIMP-1:MMP-9 complexes.

5. A method according to claim 1, wherein the TIMP-1 determined is free TIMP-1.

6. A method according to claim 1, wherein the TIMP-1 determined is the ratio between total TIMP-1 and TIMP-1:MMP-9 complex or vice versa.

7. A method according to claim 1, wherein the TIMP-1 determined is the ratio between total TIMP-1 and free TIMP-1 or vice versa.

8. A method according to claim 1, wherein the TIMP-1 determined is the ratio between TIMP-1:MMP-9 and free TIMP-1 or vice versa.

9. A method according to any of claims 1-8, wherein the body fluid is selected from the group consisting of blood (serum and plasma), urine and cerebrospinal fluid.

10. A method according to claim 9, wherein the body fluid is plasma.

11. A method according to claim 1, wherein the discrimination between high and low likelihood of having cancer is performed on th basis of the concentration of total, complexed and/or free TIMP-1 and/or on the basis of the ratios thereof.

12. A method according to claim 11, wherein the body fluid is blood, and the concentration threshold at and above which members of the population are discriminated as having a high likelihood of having colorectal cancer is 91.3 microgram/L, calculated on the basis of plasma TIMP-1 measurements in healthy blood donors and in a population of colorectal cancer patients.

13. A method according to claim 11, wherein the body fluid is blood, and the concentration threshold below which members of the population are discriminated as having a low likelihood of having colorectal cancer is 91.3 microgram/L, calculated on the basis of plasma TIMP-1 measurements in healthy blood donors and in a population of colorectal cancer patients.

14. A method according to claim 11, wherein the discrimination is performed on the basis of an assessment involving the concentration of plasma TIMP-1 and the desired diagnostic sensitivity and specificity.

15. A method according to claim 11, wherein the discrimination is performed on the basis of an assessment involving the concentration of TIMP-1:MMP-9 complexes.

16. A method according to claim 11, wherein the discrimination is performed on the basis of an assessment involving the concentration of free TIMP-1.

17. A method according to claim 11, wherein the discrimination is performed on the basis of an assessment involving one or more of the ratios between total TIMP-1, TIMP-1:MMP-9 complex and/or free TIMP-1.

18. A method according to any of the preceding claims, wherein the concentration determination is performed by means of ELISA or zymografi.

19. A method according to any of the preceding claims, wherein the cancer type is selected from the group consisting of colorectal cancer, breast cancer, lung cancer, prostate cancer, ovarian cancer, cervical cancer, liver cancer and gastric cancer.

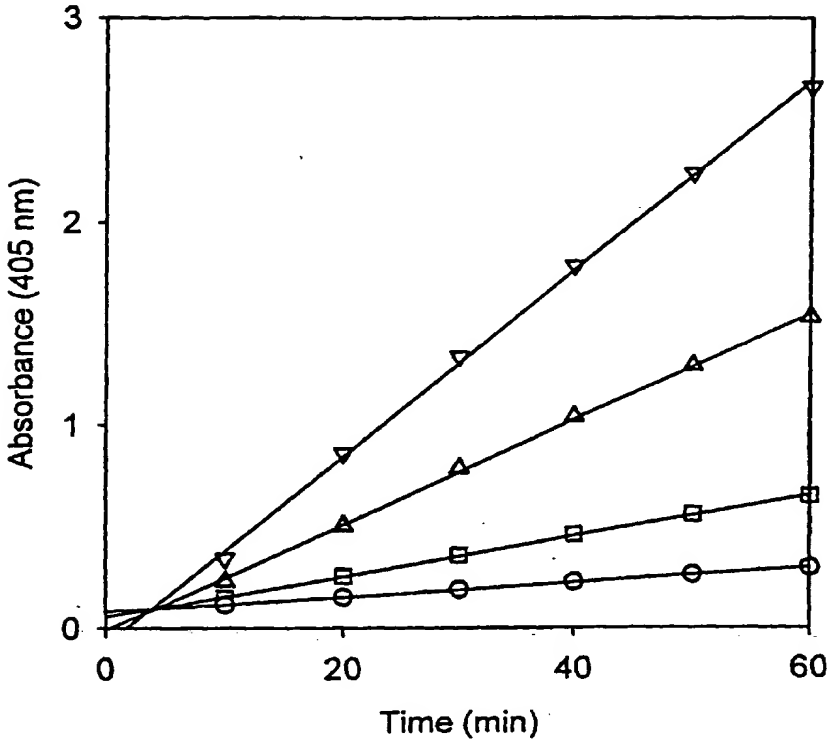


Fig. 1

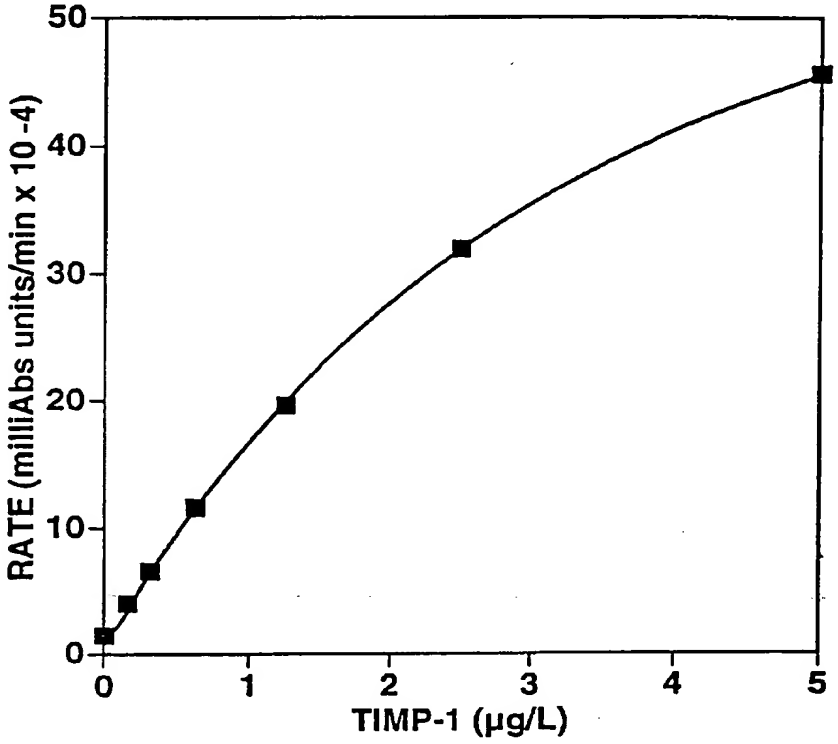


Fig. 2

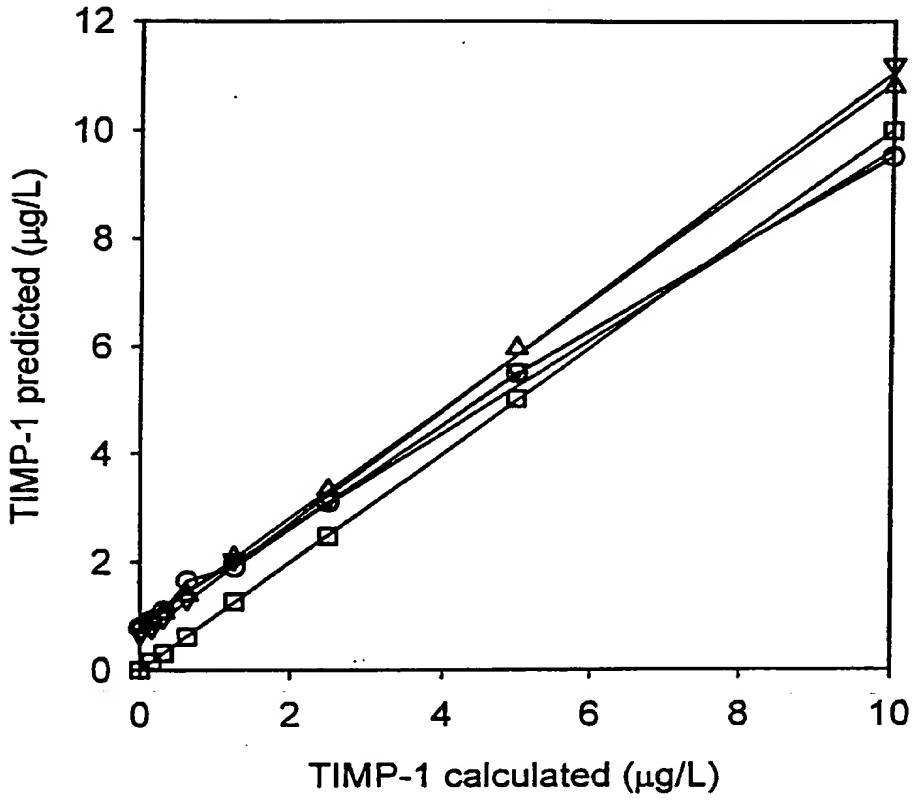


Fig. 3

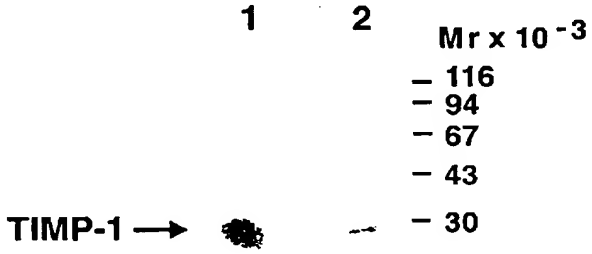


Fig. 4

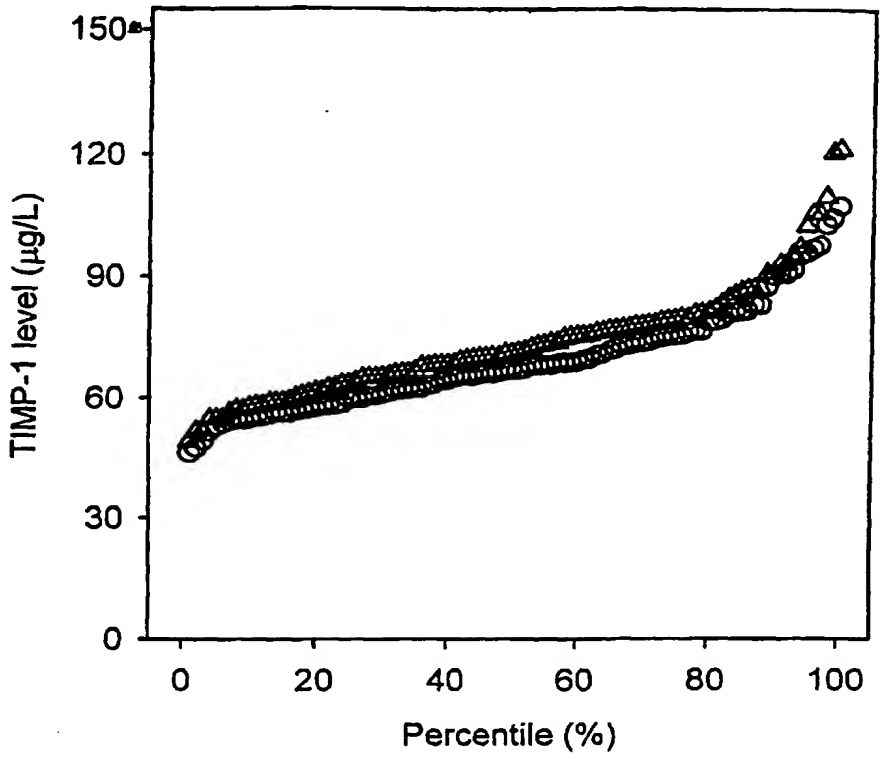

150
120
90
60
30
0
TIMP-1 level (µg/L)
0
20
40
60
80
100
Percentile (%)

Fig. 5a

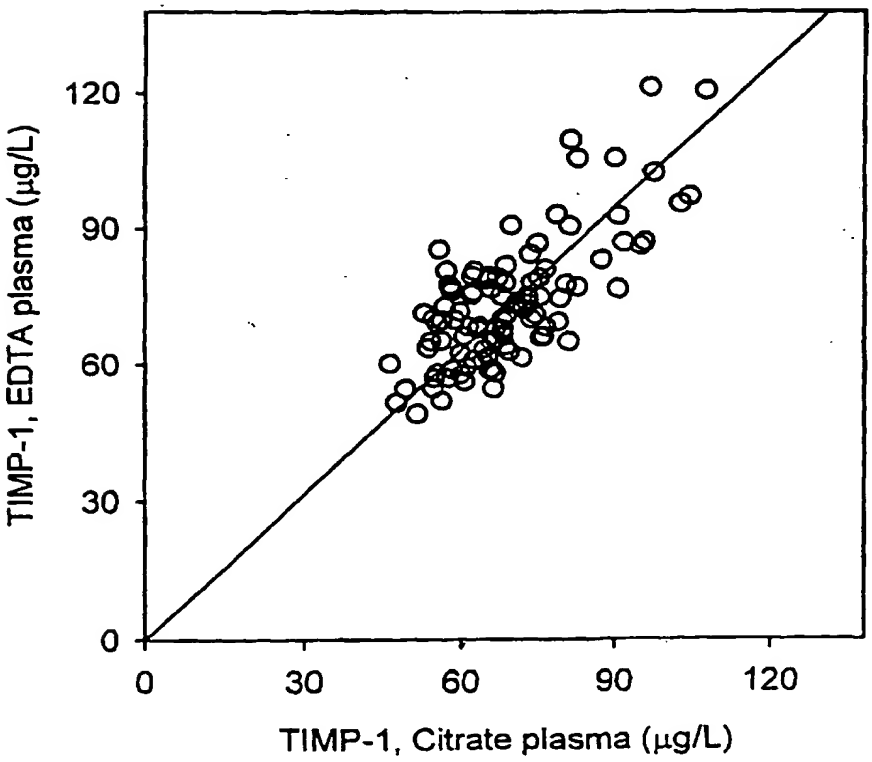

120
90
60
30
0
TIMP-1, EDTA plasma (µg/L)
0
30
60
90
120
TIMP-1, Citrate plasma (µg/L)

Fig. 5b

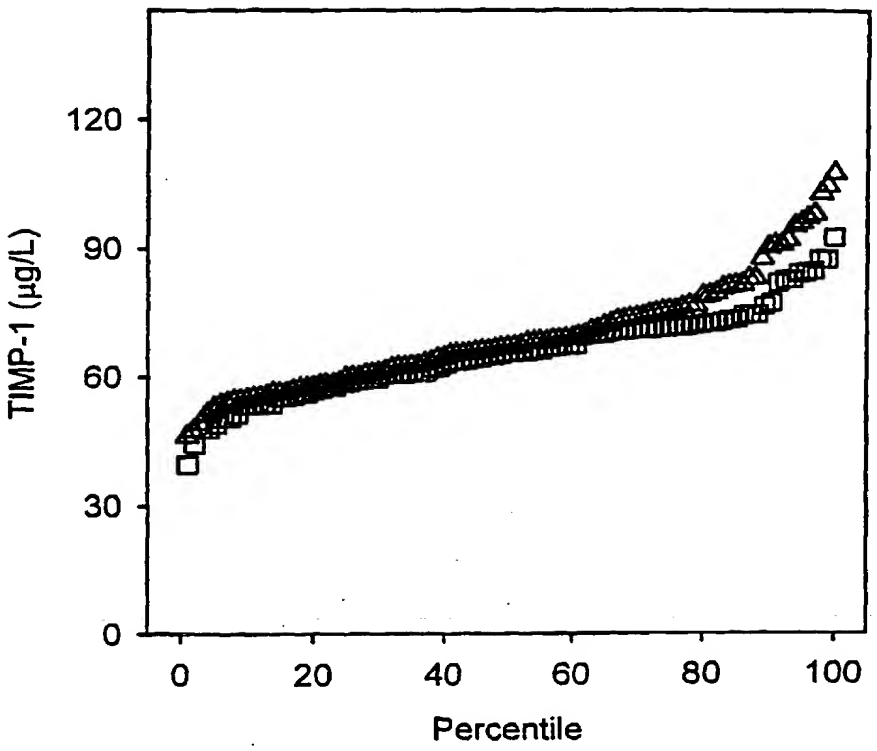


Fig. 6

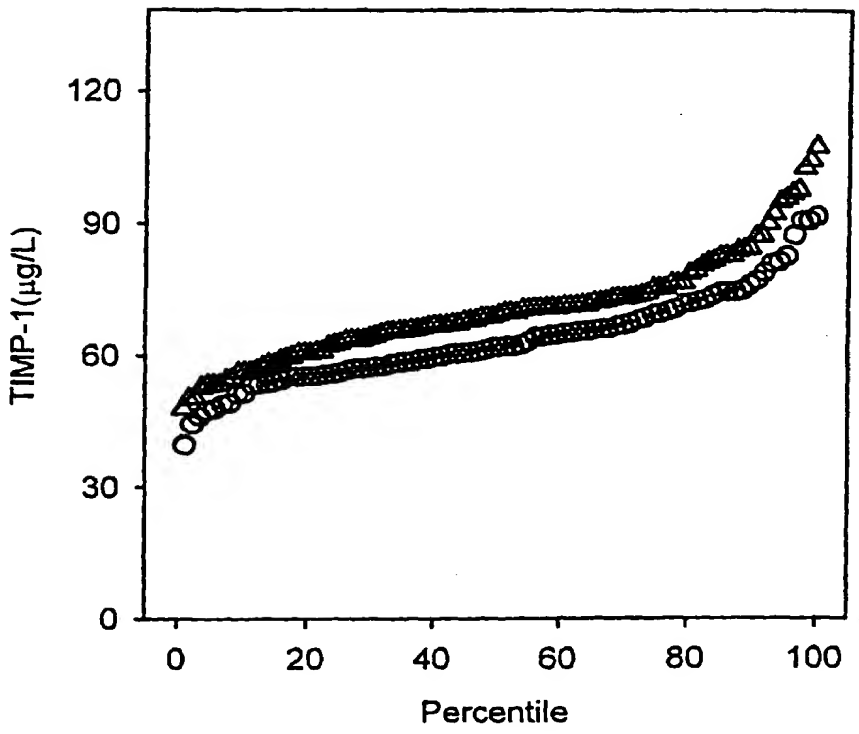
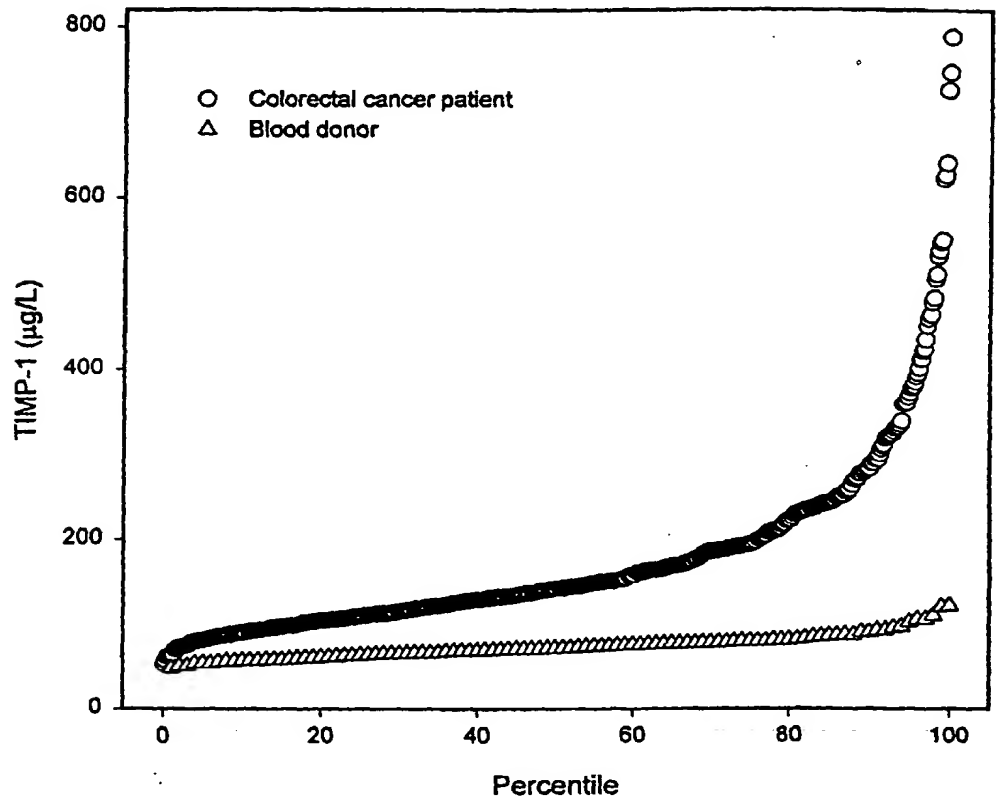


Fig. 7



Blood donor 90th centile = 91.8 µg/L
Colorectal cancer patient 10th centile = 91.1 µg/L

Fig. 8

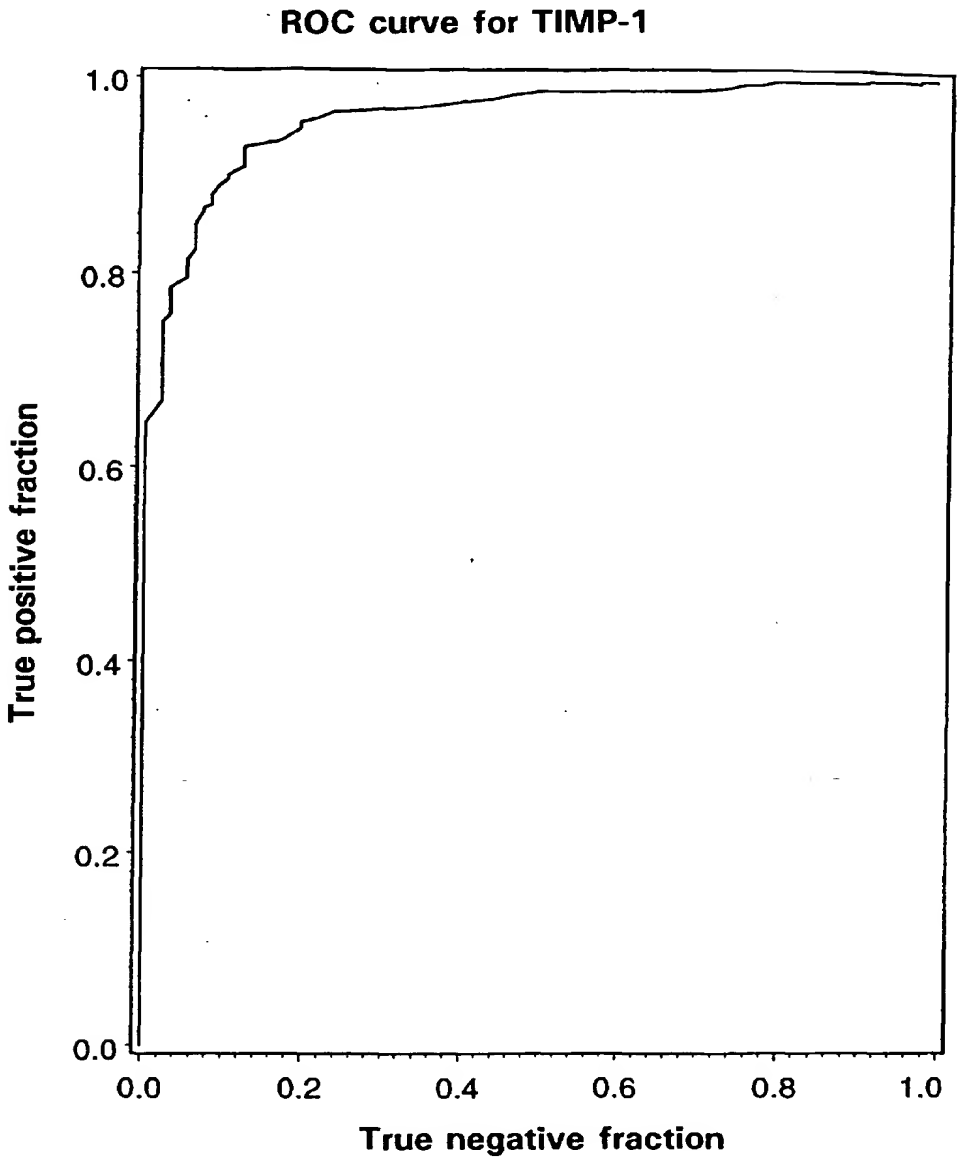


Fig. 9

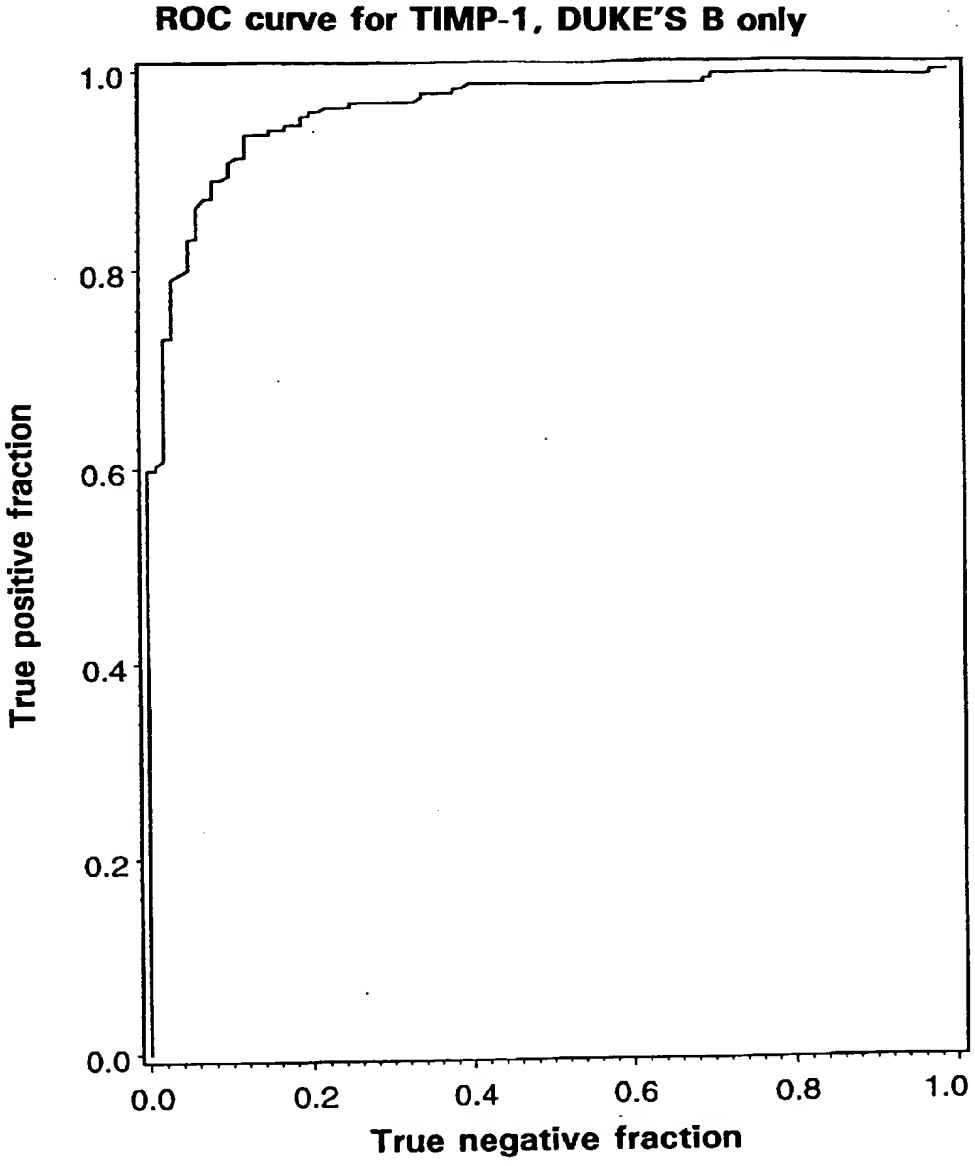

ROC curve for TIMP-1, DUKE'S B only
True positive fraction
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
True negative fraction

Fig. 10

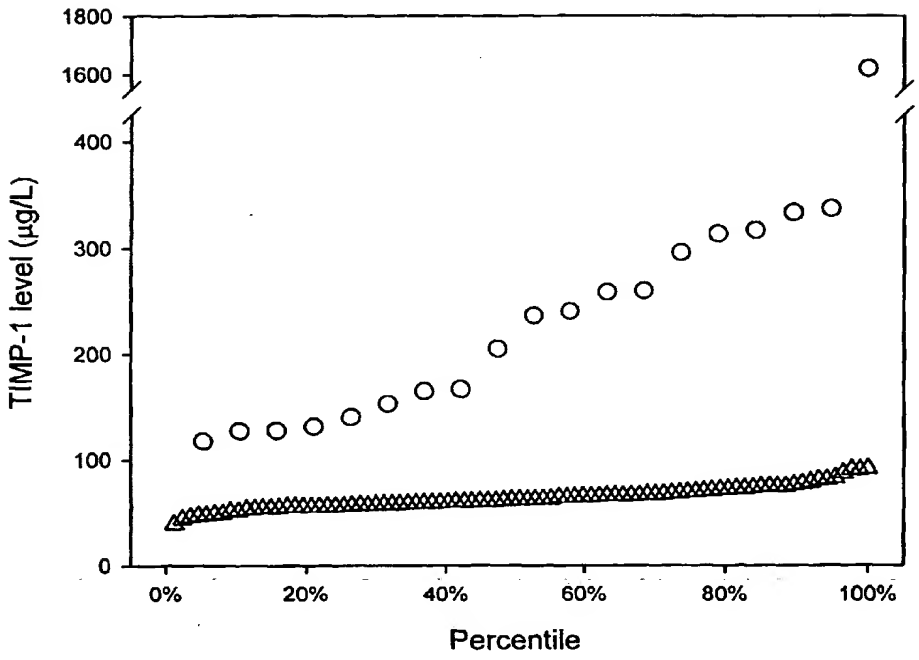

1800
1600
400
300
200
100
0
TIMP-1 level (µg/L)
0%
20%
40%
60%
80%
100%
Percentile

Fig. 11

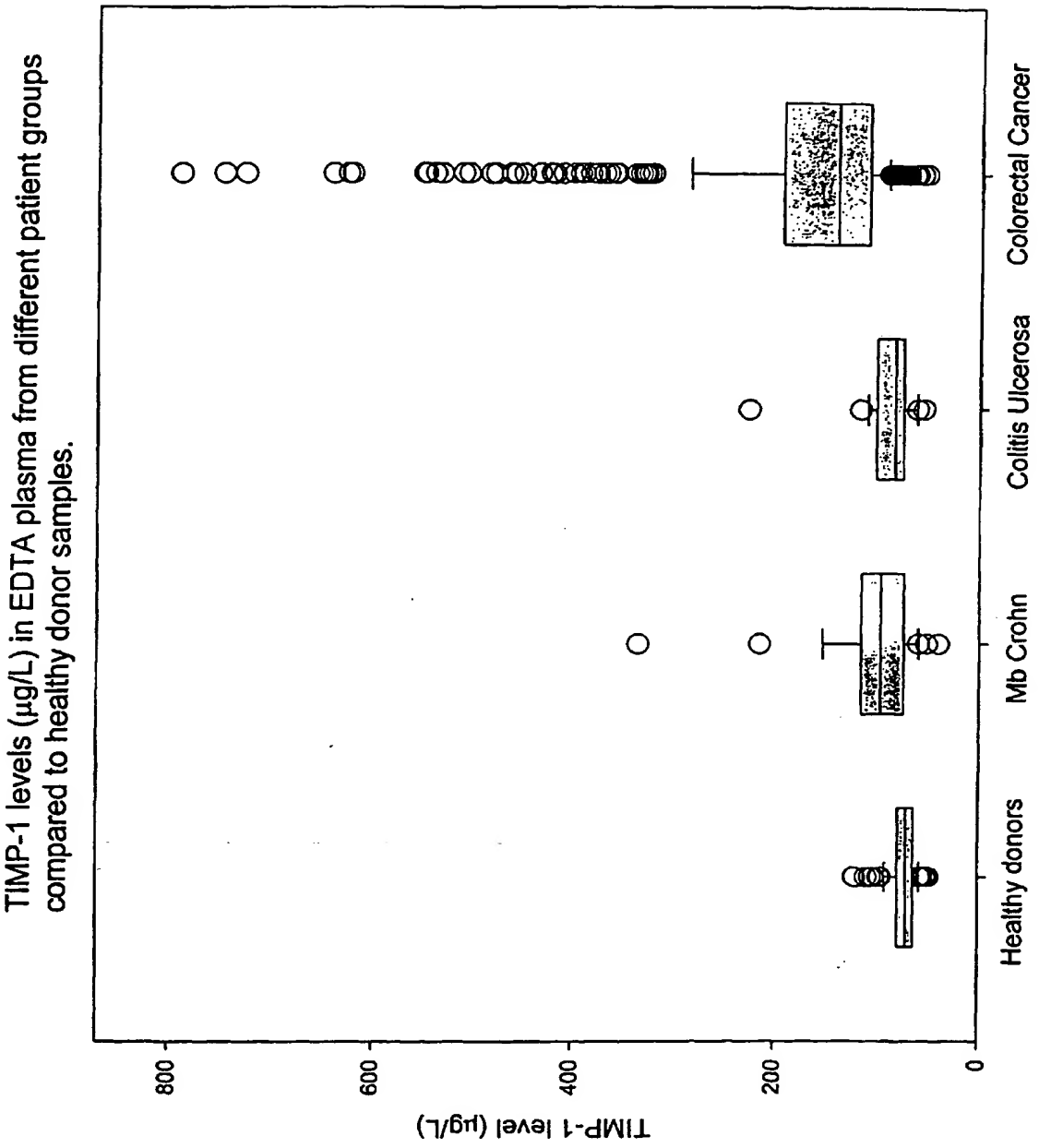

TIMP-1 levels (µg/L) in EDTA plasma from different patient groups compared to healthy donor samples.
TIMP-1 level (µg/L)
0
200
400
600
800
Healthy donors
Mb Crohn
Colitis Ulcerosa
Colorectal Cancer

Fig. 12